Table 1 Clinical and biological data of children with essential thrombocythemia

Patient No.	*Age (years)*	*Sex*	*Symptoms at Diagnosis*	*Splenomegaly*	*Platelets ($\times 10^9$/l)*	*WBC ($\times 10^9$/l)*	*HB (g/dl)*	*LAP*	*Ph′*[a]	*Treatment*	*Follow-up (years)*
1	4	F	Headache	Yes	3558	14,2	123	↓	Negative	Anagrelide	4
2	8	F	Headache	No	726	5,7	110	N	Negative	Anagrelide	2
3	7	F	No	Yes	800	11	134	N	Negative	None	3
4	3	M	No	No	1400	15	125	N	Negative	Anagrelide	5
5	15	F	No	No	1101	6,5	142	N	Negative	Anagrelide	16

[a] Ph′, Philadelphia chromosome studied by conventional cytogenetics and fluorescence *in situ* hybridization with BCR/ABL probe. N, normal; ↓, low; LAP, leukocyte alkaline phosphatase.

Table 2 *In vitro* cultures of circulating myeloid progenitor cells in pediatric essential thrombocythemia

Patient No.	*No. BFU-E*	*No. BFU-E endogenous*	*No. CFU-MK*	*No. CFU-MK endogenous*	*No. CFU-GM*	*No. CFU-GM endogenous*
1	128	2	6	2	46	0
2	80	0	8	0	78	0
3	120	6	3	3	46	0
4	24	8	6	5	6	0
5	40	8	10	4	6	0
Total (mean ± s.d.)	78.4 ± 41	4.8 ± 3.2	6.6 ± 2.3	2.8 ± 1.7	36.4 ± 27.4	0
CV (mean ± s.d.)	34 ± 12	0	1 ± 1.2	0	10 ± 3	0 ± 0

No., number of progenitors/2 × 10^5 mononucleated cells; s.d., standard deviation; CV, control values.

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Identification and characterization of rapidly dividing U937 clones with differential telomerase activity and gene expression profiles: role of c-Myc/Mad1 and Id/Ets proteins

Leukemia (2002) **16,** 1877–1880. doi:10.1038/sj.leu.2402607

TO THE EDITOR

The human leukemic U937 cell line has been widely used to study neoplasia, cancer therapeutics, cell differentiation, and HIV infections. It has been previously found by limiting dilution cloning that some U937 subclones (plus clones) support HIV replication efficiently and some (minus clones) do not.[1] Based on the more mature phenotype of the minus clones and observations that telomerase activity declines during cell maturation in humans[2] we hypothesized that they are in a more 'differentiated' state than the plus clones and therefore their telomerase activity would be lower than that of the plus cells. Here, we report the striking difference between plus and minus U937 clones in their telomerase activity, telomere length, apoptosis, growth rate and gene expression, but similarity in the rate of their division as measured by BrdU incorporation (Tables 1 and 2). The results suggest possible mechanism for differential regulation of telomerase activity and cell division in these cells, a potential role for Ets1 and Ets2 in telomerase activity regulation, and the existence of another yet to be identified pathway(s) of Id2 and telomerase regulation, which is currently under investigation. Thus minus and plus clones could be used as a model system to study regulation of telomerase and other differentiation-related genes in leukemic cells, and their gene expression profiling could help in elucidation of the role of telomerase and other cancer-related genes for progression of cancer in leukemias.

In an attempt to elucidate the mechanism of differential telomerase activity regulation in the two subclones we evaluated the role of c-Myc/Mad1 by Western blotting and RT PCR. We found positive correlation of telomerase activity with c-Myc/Max expression and negative with Mad1 (Table 1). Furthermore, differentiation agents modulating c-Myc level altered telomerase activity in a parallel fashion, suggesting a role for the c-Myc pathway in telomerase regulation (data not shown). This is in agreement with data for HL60 cells[3] demonstrating the importance of the c-Myc/Max/Mad1 interplay for telomerase regulation in leukemia cell lines.

To find other genes that are differentially expressed in the minus and plus clones, and may have a role in the regulation of cell growth and telomerase activity, we measured the gene expression profiles of minus and plus clones. The samples were duplicated and only those 684 genes that have statistically significant differences (*t*-test, $P < 0.05$) and positive calls (P following the Affymetrix notation for

Correspondence: DS Dimitrov, LECB/NCI-Frederick, NIH, Bldg 469, Rm 246, PO Box B, Miller Drive, Frederick, MD 21702-1201, USA; Fax: 301–846–6189
Received 22 November 2001; accepted 15 April 2002

Table 1 Characterization of plus and minus clones

	Plus	*Minus*
Parameters		
Telomerase activity, ratio	100	1
Telomere length, kb	20	3.5
Apoptotic index, %	3.3	8.7
BrdU labeling, %	64	70
Growth rate, ratio	2	1
Relative expression		
c-Myc	2	1
Mad1	1	3
c-Myc/Max	>10	1
Ets 1/2	2–3	1
Fli1	1	3
Id1	1	3
Id2/Id3/Id4	1	>10

Telomerase activity was measured by the PCR TRAP assay, telomere length – by gel electrophoresis, apoptosis and BrdU labeling – by flow cytometry, growth rate – by counting number of cells as function of time microscopically, and the protein expression – by Western blotting.

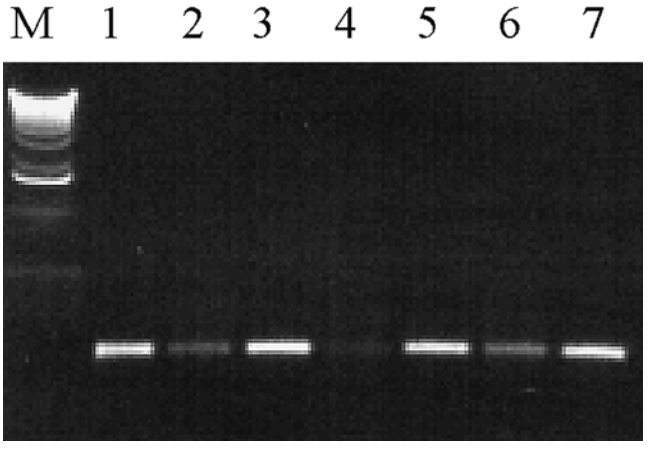

Figure 1 PCR-based protein/DNA interaction showing specific Ets2 binding to hTERT promoter. Ets proteins were immunoprecipitated from cell lysate from 1×10^7 cells by using 5 μg of anti-Ets antibody mixed with 2 μl of protein G beads. The hTERT promoter was then added to the immunoprecipitation product in EMSA buffer. After washing, promoter fragments bound as such were eluted and subjected to PCR amplification and agarose gel analysis. Lane 1, 1% hTERT promoter input used as PCR positive control. Lane 2, BSA used in place of Ets2 antibody as negative control. Lane 3, sample from plus cell lysate. Lane 4, same as lane 3 but with peptide that was used to generate Ets2 antibody added as competitor. Lane 5, sample from minus cell lysate. Lane 6, same as lane 3 but with oligonucleotide containing Ets consensus binding site added. Lane 7, same as lane 3 but with oligonucleotide that is different from consensus binding site by 1 base pair added. M-1 kb DNA molecular weight marker.

presence) for the two replicates of one of the clones were considered. Of these genes 27 and 14 were more than five-fold (Table 2), and 156 and 238 genes were more than two-fold up-regulated or down-regulated in clone 17 relative to clone 10, respectively. The differentially regulated genes are involved in a variety of cell functions including proliferation, differentiation and apoptosis, cytoskeletal organization, enzymatic activities and signaling through receptors (Table 2). The mRNA of c-Myc, Mad1 and Sp1 was about two- to three-fold differentially expressed in agreement with the results from the Western blotting and mRNA RT PCR. The level of hTERT mRNA was below the level of detection of the oligonucleotide microarray for both plus and minus cells. Three of the genes which were previously shown to be highly expressed in the minus clones,[1] cathepsin G, elastase and proteinase 3, were absent in the plus cells and highly expressed in the minus cells (above 1000 relative units). These results demonstrate that for these particular clones and genes the microarray data are in agreement with the data obtained by Western blotting and mRNA PCR. Of those genes which are detectable in both plus and minus cells the most highly up-regulated gene in the minus clones was Id2 – about 50-fold. These results suggested a role of Id proteins in the distinct properties of minus cells.

Id proteins do not bind DNA directly but often participate actively in transcriptional regulation by interacting with members of the Ets family proteins. More than 30 Ets members have been identified that regulate directly more than 200 targets involved in multiple cellular events, including cell differentiation and proliferation,[4] and cell senescence through the p16-dependent pathway.[5] To find whether c-Myc and Ets proteins regulate telomerase expression through direct association with its promoter or by indirect mechanisms we used an EMSA assay and a PCR-based methodology. By using the EMSA assay we did not observe binding of c-Myc to the hTERT promoter although c-Myc was abundant in nuclear extracts from plus cells (data not shown). This was not surprising because other investigators also failed to detect c-Myc-hTERT promoter interactions by EMSA except for an *in vitro* expressed c-Myc bound to DNA with the addition of purified Max;[6] in addition, c-Myc bound to the hTERT promoter[7] was detected by CHIP. However, another transcrpition factor, Sp1, bound specifically to the hTERT promoter, as shown by the super-shift created with an anti-Sp1 antibody (data not shown). There was significantly more Sp1 binding in nuclear extract from plus cells than from minus cells (data not shown) which correlated with the amount of Sp1 estimated by Western blotting. This suggested that the EMSA assay was working properly but the failure to detect c-Myc binding was likely due to its weak association with both Max and DNA leading to its disassociation from Max and eventually DNA during the gel analysis step. To detect the c-Myc–DNA interaction for our experimental system we used a sensitive PCR-based assay that does not involve gel electrophoresis steps (Xiao and Dimitrov, in preparation). Because immunoprecipitated c-Myc remains associated with Max in very mild lysis buffer, it should retain its native conformation and DNA-binding ability. The bound DNA then can be amplified by PCR amplification and detected by standard agarose gel analysis. By using this assay we demonstrated direct association of the hTERT promoter with c-Myc and Sp1 in lysates from plus cells, and Mad1 in lysates from minus cells (data not shown). The respective binding of c-Myc and Sp1 in lysates from minus and Mad1 in lysates from plus cells was significantly lower. Weak binding by Ets1 and Ets2 was also detected, but there was no significant difference in their binding to hTERT promoter between plus and minus cells. Figure 1 shows specific Ets2 binding to the hTERT promoter. Different antibodies, irrelevant protein (BSA), and cell lysates from different cell lines were used as negative controls. Whenever possible, binding by *in vitro* expressed and purified protein was used to validate the data from the PCR-based assay as, eg, for Ets1 and Ets 2 (data not shown). These data provided evidence that direct association of c-Myc, Mad1 and Sp1 with the hTERT promoter is a likely mechanism of telomerase regulation in U937 cells. The finding that Ets1 and Ets2 bind to the hTERT promoter also suggests that these transcription factors might be involved in telomerase activity regulation, but are unlikely to be responsible for the major differences between plus and minus cells.

The identification of U937 clones with strikingly different telomerase activity, and differential expression of Id and Ets family proteins but similar division rates provided a unique model system to study regulation of telomerase, and other differentiation and cancer-related genes. We provided evidence that c-Myc/Mad1 and Sp1 are likely the major factors regulating the telomerase activity in these subclones, and demonstrated a possible molecular mechanism involving their direct binding to the telomerase promoter. We also found that Ets1 and Ets2 bind specifically to the hTERT promoter suggesting possible role of these protein families in regulating telomerase activities. Even though Ets1 and Ets2 might not contribute to the striking different telomerase activities in the plus and minus subclones, we can not rule out the potential role of other Ets family members due to their differential binding affinity to EBS within hTERT, and to Id family proteins. A general picture is emerging about the complex, in many cases redun-

Table 2 Differential gene expression in U937 minus and plus cells measured by the Affymetrix 12k human genome microarray (HG U95a)

ATPase inhibition					
S100A11	[D38583]	(0.13)			
Cell proliferation and differentiation					
IGFBP2	[S37730]	(12.0)	*PLP2*	[U93305]	(0.12)
KIAA0128	[D50918]	(9.4)	*NDRG1*	[D87953]	(0.07)
ISG15	[AA203213]	(7.3)	*TIMP1*	[D11139]	(0.07)
EMP3	[U87947]	(0.19)	*EVI2B*	[M60830]	(0.06)
GRN	[AF055008]	(0.19)	*Id2*	[D13891]	(0.02)
LGALS1	[AI535946]	(0.17)	*Id1*	[X77956]	(<0.1)
Chaperones					
XPNPEP2	[AL023653]	(11.0)	*RP2*	[AJ007590]	(0.18)
HSPCA	[X15183]	(5.2)	*IFI30*	[J03909]	(0.17)
Cytoskeletal organization					
DOC1	[AF006484]	(6.6)	*CD2AP*	[AL050105]	(5.5)
DNA repair					
TDG	[U51166]	(5.4)			
Histocompatibility					
HLA-C	[X58536]	(0.07)			
Hypothetical proteins with unknown function					
753P9	[AL023653]	(11.0)	*TNRC3*	[AI743134]	(0.17)
KIAA0974	[AI680675]	(6.0)	*KIAA0856*	[AB020663]	(0.14)
LOC56267	[AF091090]	(5.2)	*DKFZP586F2423*	[AL080209]	(0.12)
TNRC5	[U80744]	(0.18)			
Metabolic enzymes					
PP15	[X07315]	(10.0)	*BCKDHA*	[Z14093]	(0.12)
OXCT	[U62961]	(6.6)	*P4HB*	[M22806; J02783]	(0.10)
TIMP1	[D11139]	(0.7)	*PRG1*	[X17042]	(0.05)
Phospholipid transport					
PLTP	[L26232]	(0.16)			
Potassium channels					
KCNAB2	[AF044253]	(0.19)			
Proteases					
CTSG	[J04990]	(>50)	*PRTN3*	[M29142]	(>50)
ELA2	[D00187]	(>50)	*CTSD*	[M63138]	(0.11)
Receptors					
CRY1	[D83702]	(7.7)	*FCGRT*	[U12255]	(0.12)
TFRC	[X01060]	(5.6)			

The ratio of signals from plus to minus cells is in ( ) parentheses. The Genebank accession numbers are shown in parentheses. All gene names are from the GeneCard database.

dant, interrelationships between numerous regulators, which can affect U937 cell fate in multiple ways even for very similar clones.

Acknowledgements

We wish to thank Gina Cremona for the help with preparation of Table 2.

X Xiao[1]
SK Phogat[1]
IA Sidorov[1]
J Yang[2]
I Horikawa[3]
D Prieto[2]
J Adelesberger[2]
R Lempicki[2]
JC Barrett[3]
DS Dimitrov[1]

[1]*Laboratory of Experimental and Computational Biology, National Cancer Institute at Frederick, NIH, Miller Drive, Frederick, MD, USA;* [2]*SAIC-Frederick, Frederick, MD, USA;* [3]*Laboratory of Biosystems and Cancer, National Cancer Institute, NIH, Bethesda, MD, USA*

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Liposomal daunorubicin (DaunoXome) for treatment of relapsed meningeal acute myeloid leukemia

Leukemia (2002) **16,** 1880–1881. doi:10.1038/sj.leu.2402617

TO THE EDITOR

Expression of multidrug resistance (MDR) proteins is a major obstacle in the treatment of acute myeloid leukemia (AML). In fact, the MDR-phenotype has been associated with lower remission rates and shorter overall survival in AML patients.[1] Central nervous system (CNS) involvement is also currently considered as an adverse prognostic factor for AML, since conventional therapies are poorly effective and new strategies are not clearly identified.

Acute promyelocytic leukemia, AML-M3 subtype or APL, characterised by anomalies of the α-*trans*-retinoic acid receptor, is the only AML subtype that never presents MDR over-expression. This form is particularly sensitive to the combination of idarubicin (Ida) and α-*trans*-retinoic acid (ATRA), with very high complete remission (CR) rates.[2] The rare cases resulting refractory to induction therapy are characterised by poor prognosis, as alternative treatments have, up to now, not been established.

Liposomal daunorubicin (DaunoXome or DNX®, Gilead Science, Foster City, CA, USA) has shown more efficacy than free daunorubicin against multidrug resistance cell lines,[3] lower toxicity profile[4] and, in particular, capability to overcome the blood–brain barrier. We thus administered DNX plus cytosine-arabinoside to an early relapsed MDR+ AML and to an APL refractory patient, both with meningeal involvement.

The first case refers to a 36-year-old female, diagnosed for AML M4 subtype in March 2000. Two adverse prognostic factors were present at diagnosis: hyperleukocytosis (200×10^9/l) and MDR over-expression, tested by flow cytometry using a panel of monoclonal antibodies (MRK-16, LRP-56 and MRPm6, Valtex Occhiena for Novartis). CR was obtained with FLAI-5 scheme (fludarabine 25 mg/m^2/day, days 1–5; cytarabine 2000 mg/m^2/day, days 1–5 and mitoxantrone 10 mg/m^2/day, days 1, 3, 5), then reinforced with two courses: high-dose cytarabine (3 g × 2/m^2/day for 6 days) and MEC-4 scheme (mitoxantrone 12 mg/m^2/day, days 1–4; etoposide 100 mg/m^2/day, days 1–4 and cytarabine 1000 mg/m^2/day, days 1–4). Six months later, a relapse was documented, involving bone marrow and CNS. A nuclear magnetic resonance (NMR) documented an atypical localisation involving only the cerebellar leptomeninges. Three therapeutic lumbar punctures did not reveal clear fluid from the leukemic cells. Thus, DaunoXome (100 mg/m^2/day, days 1–3) plus cytarabine (2000 mg/m^2/day, days 1–5) was administered. NMR and bone marrow aspirate documented a CR, on day 14 and 21 from the end of chemotherapy respectively, still persisting after 13 months.

The second case refers to a 37-year-old male, diagnosed for APL. Hyperleukocytosis (70×10^9/l) was present at diagnosis. Induction regimen, based on idarubicin and ATRA, followed by three consolidation courses (induction therapy: 45 mg/m^2/day ATRA were administered orally until the achievement of CR or for a maximum of 90 days, with four 12 mg/m^2 doses of Idarubicin given intravenously (i.v.) on days 2, 4, 6 and 8; consolidation: AraC 1 g/m^2 and Idarubicin 5 mg/m^2 for 4 days (first cycle); mitoxantrone 10 mg/m^2 and etoposide 100 mg/m^2 for 5 days (second cycle); idarubicin 12 mg/m^2 for 1 day, subcutaneous AraC 100 mg/m^2 every 8 h and oral administration of 6-thioguanine 70 mg/m^2 every 8 h for 5 days (third cycle)) was administered. Morphological and karyotypical complete remissions were obtained. On the contrary, RT-PCR showed persistence of disease. While on therapy, 12 months from diagnosis, a CNS involvement was documented. Daunoxome (100 mg/m^2, for 3 days) plus cytarabine (2000 mg/m^2, for 5 days) were administered, inducing molecular CR.

After induction therapy, stem cell transplantation was performed, as both patients were in CR. The first one is still in CR (13 months); the second one died from infection, still in CR, after 4 months.

Anthracyclines are the reference drugs for treatment of AML, but a few factors limit their use and effectiveness. One element is toxicity, especially mucosal (oral and intestinal) and cardiac. This factor limits the administration of the drug particularly in relapsed or resistant patients, heavily pre-treated or patients who are candidates for BMT. In addition, the over-expression of MDR proteins reduces the intracellular concentration of anthracyclines, that are pumped out across the cell membrane. Moreover, these drugs do not reach adequate concentrations in the central nervous system (CNS), being ineffective against disease at this level. In our first report, the patient presented a very atypical localisation. In fact AML blasts can involve CNS in about 10% of cases at relapse, but isolated cerebellar leukemic meningitis has not been definitely described. DaunoXome is a special liposomal formulation of daunorubicin, entrapped into small liposomes. This combination increases blastic cells exposure by protecting the intracellular drug from transporter proteins. This leads to lower toxicity against normal tissues and increases efficacy against tumor cells. Furthermore, DNX overcomes the blood–brain barrier, being effective also in CNS diseases such as gliomas/glioblastomas, astrocytomas, medulloblastomas, ependymomas, craniopharyngiomas and lymphomas.[5] Only few data are available regarding the efficacy of DNX in poor risk acute leukemia[6] and, in particular, no data about DNX effectiveness against CNS myeloid leukemia have been reported before, so this is the first demonstration of DNX efficacy in CNS AML. Moreover, this is the first report regarding effectiveness of DNX in APL and its ability to induce molecular remissions in ATRA resistant patients as well.

We conclude that DaunoXome seems thus to be a useful drug for poor risk AML with CNS involvement. Further studies are warranted in order to establish its definitive role.

Acknowledgements

This work was supported in part by MURST ex 60% (S Tura) and FONDI ex 40% (S Tura).

PP Piccaluga[1]
G Visani[2]
G Martinelli[1]
A Isidori[1]
M Malagola[1]
M Rondoni[1]
M Baccarani[1]
S Tura[1]

[1] *Institute of Haematology and Clinical Oncology 'L and A Seràgnoli' University of Bologna Bologna, Italy; and*
[2] *Department of Hematology S Salvatore Hospital, Pesaro, Italy*

Correspondence: P Paolo Piccaluga, Institute of Haematology and Clinical Oncology 'L and A Seràgnoli', University of Bologna, S Orsola Hospital, via Massarenti 9, 40138 Bologna, Italy; Fax: 39 051 636 4037

Received 29 January 2002; accepted 22 March 2002